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Chromium accumulation by two *Streptomyces* spp. isolated from riverine sediments

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Strains designated R22 and R25, isolated from Salí River sediments, Argentina, were highly resistant to chromium. These strains were shown by 16S rRNA sequencing studies to be *Streptomyces* spp.; this affiliation was consistent with morphological and chemical characteristics. Growth of strains R22 and R25 in medium containing 100 mg l^{-1} chromate was reduced by only 23% and 34%, respectively, compared with growth in medium without added chromium. *Streptomyces* sp. strains R22 and R25 both accumulated chromium with yields of 10.0 and 5.6 mg Cr g^{-1} of dry weight, respectively, and a chromate concentration of 50 mg ml^{-1}. Cell fractionation studies with strain R22 showed that the great majority of the chromium were associated with the cell wall fraction. *Streptomyces* strains R22 and R25 may have applications in bioremediation of chromium contamination. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 210–215.

Keywords: *Streptomyces*; chromium accumulation; metal resistance; 16S rRNA

Introduction

The main industrial uses of chromium are in the manufacture of metallic alloys for structural and protective purposes, in fabric dyes, and in the tanning of leathers. Chromium is usually found in nature in its trivalent state, but hexavalent chromate is found generally as a result of human activities. In an environmental context, accelerating pollution by toxic metals, metaloids, radionuclides, and organometaloids has stimulated research in the use of microorganisms for metal removal and/or recovery from the biosphere [32].

High concentrations of chromate in the environment are toxic for microorganisms [1]. Mechanisms of microbial chromate resistance include reduced intracellular accumulation by modification of specific metal carriers or by possession of a highly efficient efflux system and chemical reduction of Cr(VI) to Cr(III). Studies of chromate resistance in microorganisms have generally focused on Gram-negative bacteria such as *Alcaligenes*, *Enterobacter*, and *Pseudomonas* where resistance is due to reduced intracellular accumulation of chromate [18]. Reduction of chromate [Cr(VI)] to less toxic chromium [Cr(III)] by cell cultures and/or cell-free extracts has been found in Gram-negative bacteria [17,34,36] and in Gram-positives, including one *Streptomyces* sp. [11], but the systems were not characterized in detail [18].

Metal-resistant actinomycetes have been isolated from several aquatic environments [4,25] and may have application in bioremediation of metal-contaminated sediments. In a previous screening program, six actinomycete strains resistant to cadmium, copper, and mercury were isolated from contaminated aquatic sediment of the Salí River in Tucumán, Argentina. These isolates were capable of sorption of more than 98% of the initial metal concentrations of cadmium and copper [4]. The aim of this study was to determine the chromium resistance of these previously isolated actinomycete strains and to investigate the subcellular compartmentalization of chromium.

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Materials and methods

Strains and media

Six actinomycete strains were originally isolated from a sediment sample obtained from the Salí River in the Río Hondo reservoir, using a sediment grab sampler. The isolation procedure is given in Amoroso *et al.* [4]. A metal-sensitive strain, the laboratory strain *Streptomyces lividans* TK24, was used as a control strain in all experiments and was shown on initial testing to be sensitive to 20 mg ml^{-1} chromate. The medium used for cultivating the cells was starch–casein (SC) agar that contained (per liter): starch 10.0 g; casein 1.0 g dissolved in 0.3 M NaOH; K_2HPO_4 0.5 g; and agar 15.0 g. The pH of the medium was adjusted to 7.0 by addition of dilute HCl prior to sterilization. Spore stocks were prepared from cultures grown on SC medium plates, as described by Hopwood *et al.* [16].

Chemo-taxonomic analysis

Actinomycete strains R22 and R25 were analyzed by thin layer chromatography (TLC) for isomeric diaminopimelic acid configurations (A2pm isomer) and for whole-cell sugar composition as described by Lechevalier and Lechavalier [23]. For morphological observations, the strains were cultured at 25°C on SC agar and on artificial soil (AS) agar [35] and microscopic examinations were carried out as described by Cross [10]. The strains were identified to generic level on morphological criteria using the guide of Lechevalier [22].

16S ribosomal RNA (rRNA) sequencing

Total DNA was prepared from strains R22 and R25 using a method modified from Ausubel *et al.* [5] as described previously [25]. Oligonucleotide primers with specificity for eubacterial 16S rRNA genes (forward primer 8–27: 5′-AGAGTTTGATCCTGGCTCAG-3′ [modified from FD1] [37] and reverse primer 1492: 5′-GGTTACCTTGTTACGACTT-3′ [29]) were used to amplify 16S rRNA genes. PCR fragments were purified using the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA) and sequenced using the PRISM Ready Reaction Kit (PE Applied BioSystems, Foster City, CA) and an ABI 373A sequencer (PE Applied BioSystems). Sequencing data were analyzed by comparison to 16S rRNA genes in the Ribosomal Database Project and EMBL-GenBank databases, and aligned manually with the Phydit software [9]. 16S rRNA gene sequences of strains R22 and R25 have been deposited in GenBank under accession nos. AF331529 and AF331830, respectively.

Evolutionary trees were inferred using the neighbor-joining [30], Fitch–Margoliash [14], and maximum parsimony [21] algorithms in the PHYLIP package [13]. Evolutionary distance matrices for the neighbor-joining and Fitch–Margoliash methods were generated as described by Jukes and Cantor [19]. The resultant tree topologies were evaluated by performing bootstrap analyses of the neighbor-joining data [12] based on 1000 resamplings. Strains used to construct the phylogenetic tree are listed in Table 1.

Plasmid screening

Strains R22 and R25 were screened for the presence of plasmids, including very large plasmids, by pulsed field gel electrophoresis (PFGE). DNA plugs for PFGE analysis were prepared by a modification of the procedure used by Kieser *et al.* [20], described in detail in Ref. [26]. Plugs were subjected to PFGE using a clamped homogeneous electric field system (CHEF DR-III; Bio-Rad, Melville, NY) in 0.5× TBE buffer (1× TBE is 98 mM Tris–HCl, 89 mM boric acid, 62 mM EDTA) containing 100 μM thiourea at 14°C [27]. Pulse time was 30–50 s for 22 h. Ladders of λ DNA concatamers (New England Biolabs, Beverly, MA) were used as molecular weight standards. DNA was stained with SYBR Green I (Molecular Probes, Eugene, OR) prior to photography with 302 nm ultraviolet light illumination using a SYBR Green gel stain photographic filter (Molecular Probes). The gels were digitized using a FluorImager 573 (Molecular Dynamics, Sunnyvale, CA).

Table 1 *Streptomyces* strains used in the phylogenetic analysis with their 16S rRNA gene accession numbers

Organism/strain	Accession number
S. lavendulae IFO 14028	D85114
S. virginiae IFO 12827	D85123
S. setonii ATCC 25497	D63872
S. bikiniensis (DSM 40581)	X79851
S. cinnabarinus ISP 5467	AJ399487
S. cyaneus ISP 5107	AJ399471
S. lincolnensis NRRL 2936	X79854
S. indigocolor ISP 5432	AJ399464
S. hygroscopicus ATCC 21431	X79853
S. galbus DSM 40089	X79852

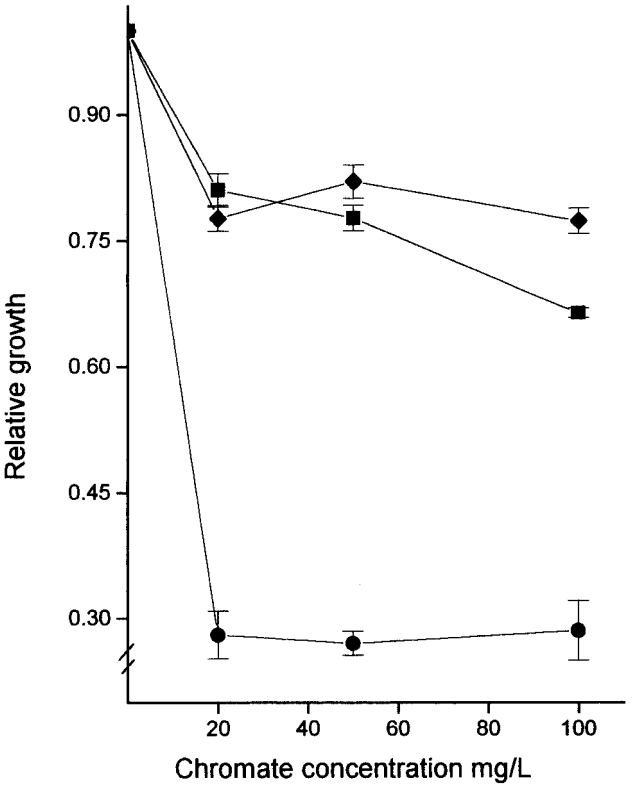

Figure 1 Effect of chromate concentration on the growth of strains R22 (◆), R25 (■), and *S. lividans* TK24 (control) (●) strains. Growth was monitored by dry weight determinations and expressed relative to growth in supplemented MM medium.

Chromium analysis

Chromium concentrations were determined by atomic absorption spectrometry. Aqueous samples were analyzed directly, whereas solid-phase (biomass) samples were first digested by dissolving pellets in concentrated nitric acid [3]. Chromium sorption to biomass was determined and calculated on a "per unit dry weight of biomass" basis. Analytical procedures for chromium were based on standard methods [3]. The amount of chromium in each subcellular fraction was also determined by atomic absorption spectrometry.

Determination of chromium toxicity

Spore suspensions of strains R22 and R25 were inoculated in a defined liquid medium (MM) containing (per liter): L-asparagine 0.5 g; K_2HPO_4 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; $FeSO_4 \cdot 7H_2O$ 0.01 g; and glucose 10.0 g (pH 7.0). The MM medium was supplemented with 20, 50, or 100 mg l^{-1} of Cr(VI) as $Na_2Cr_2O_7$ (Aldrich, WI). Cells were grown on a shaker (100 rpm) at 30°C for 72 h and centrifuged (3000×g) for 10 min. After washing the resulting pellets with 25 mM Tris–EDTA buffer (pH 8.0), the biomass was estimated by drying the pellets at 105°C to constant weight. The chromium concentration was determined in pellets and supernatants. The pellet was dissolved in concentrated nitric acid [3]. The acid solution was analyzed to determine the amount of chromium taken up by the cells. Chromium sorption yield was defined as the mass of metal per unit of biomass expressed as dry weight.

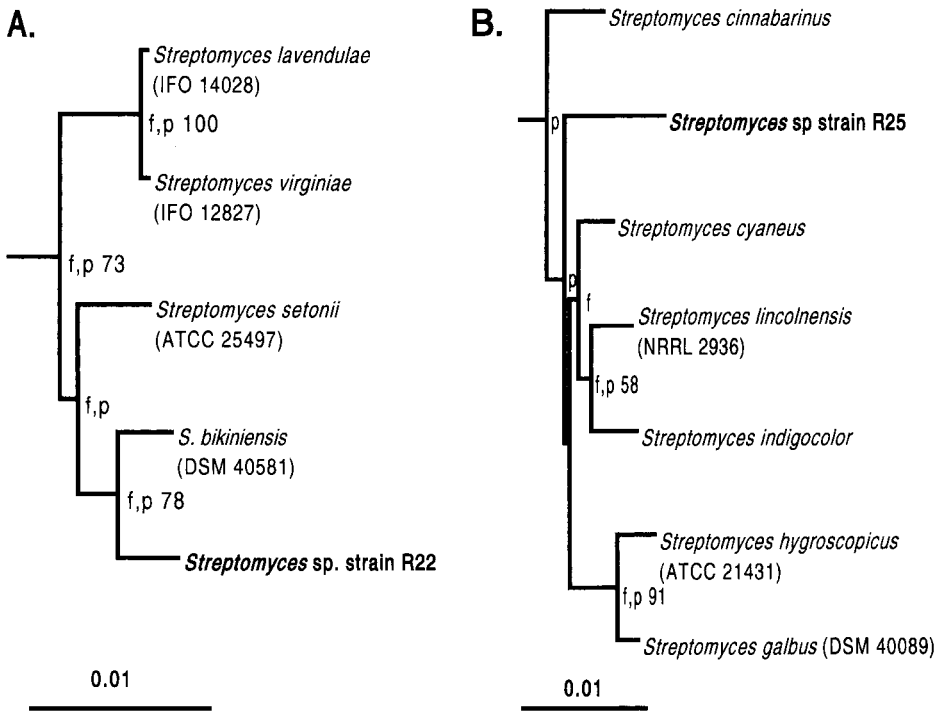

Figure 2 Phylogenetic analysis based on 16S rRNA sequence. Neighbor-joining trees for (A) strain 22 and (B) strain R25 and closely related *Streptomyces* strains are based on nearly complete (> 1400 bp) 16S rRNA sequences. (f) and (p) indicate branches that were also found using the Fitch–Margoliash or maximum parsimony methods, respectively. The numbers at the nodes are percentages indicating the levels of bootstrap support, based on a neighbor-joining analysis of 1000 re-sampled data sets. Only percentages > 50% are shown. Estimated positions of the root organisms, indicated by the outside branches, were established by including *S. coelicolor* in the analysis.

Determining subcellular chromium distributions

Strain R22 cells were grown in MM with a chromate concentration of 50 mg l^{-1} at 30°C for 72 h. Cells were washed twice and resuspended in 25 mM Tris–EDTA buffer (pH 8.0). The cell suspension was centrifuged at 33,000×*g* for 15 min to remove extracellular polymers [6]. The resultant cell pellet was resuspended in 25 mM Tris–EDTA buffer (pH 8.0) and disrupted by high pressure [33]. After cell disruption, the cell wall fraction and subcellular fractions were collected as previously described [33].

Results

Chromium toxicity

Six indigenous actinomycete strains with high tolerance to heavy metals and high metal sorption capability isolated in a previous screening program [4] were tested for growth in the presence of high concentrations of chromate. *S. lividans* TK24 was included as a negative control strain. Growth of *S. lividans* TK24 and four of the wild-type actinomycete strains was reduced by almost 70% at a chromate concentration of 20 mg l^{-1} (results not shown). However, growth of strains R22 and R25 in medium containing 100 mg l^{-1} chromate resulted in only a 23% and 34% growth decrease, respectively, relative to medium with no added chromate (Figure 1). Strains R22 and R25 were therefore selected for further study.

Morphological characteristics

Strain R22 showed the following morphological characteristics: aerial mycelium gray ("cinereus"); substrate mycelium brownish; a slight brownish extracellular water-soluble pigment was produced, long spore chains were formed in bundles, straight to flexuous ("rectus flexibilis"). Strain R25 exhibited gray brownish aerial mycelium, brown substrate mycelium, and long spore chains formed in ramificated, loose bundles. Both strains contained diaminopimelic acid based on chemical analysis of whole cell extracts. Glucose and ribose were present in their cell walls. These findings are consistent with grouping of strains R22 and R25 as *Streptomyces* spp. [22].

16S rRNA sequence analysis

More than 1400 bp of the 16S rRNA genes of strains R22 and R25 were sequenced. Analysis of the 16s rRNA sequences confirmed the grouping of strains R22 and R25, indicated by morphological characteristics, in the *Streptomyces* clade (Figure 2). *Streptomyces* sp. strain R22 was closely related to *S. bikiniensis* (Figure 2A). The closest relatives of *Streptomyces* sp. strain R25 were *S. cinnabarinus*, *S. cyaneus*, *S. lincolnensis*, *S. indigocolor*, *S. hygroscopicus*, and *S. galbus* (Figure 2B).

Plasmid screening

Two large plasmids, *ca.* 40 and 120 kb, were detected in strain R22 and no plasmids were detected in strain R25 (Figure 3).

Chromium uptake and subcellular distribution

More than 99% of chromate ions was sequestered from culture media and accumulated by *Streptomyces* sp. strains R22 and R25 in cultures with initial concentrations of chromate between 20 and 100 mg l^{-1} (data not shown). Strain R22 grown at a chromate

concentration of 50 mg l^{-1} metal resulted in a chromium yield of 10.0 mg Cr g^{-1} of dry weight (Figure 4). At 20 and 100 mg l^{-1} chromate, yields were 49% and 43%, respectively, of this maximum yield (Figure 4).

Strain R25 gave a consistent yield of between 5.6 and 7.1 mg Cr g^{-1} of dry weight in cultures with chromate concentrations between 20 and 100 mg l^{-1}. This yield was *ca.* 30% lower than the maximum yield obtained with strain R22 at a concentration of 50 mg l^{-1} (Figure 4).

Cell fractionation studies were done with strain R22 grown at a chromate concentration of 50 mg l^{-1} and gave the following yields: 94.7% chromium associated with the cell wall fraction, 2.7% with the membrane fraction, 1.6% with the exopolymer fraction, and the remaining 1.0% associated with the soluble fraction (Figure 5).

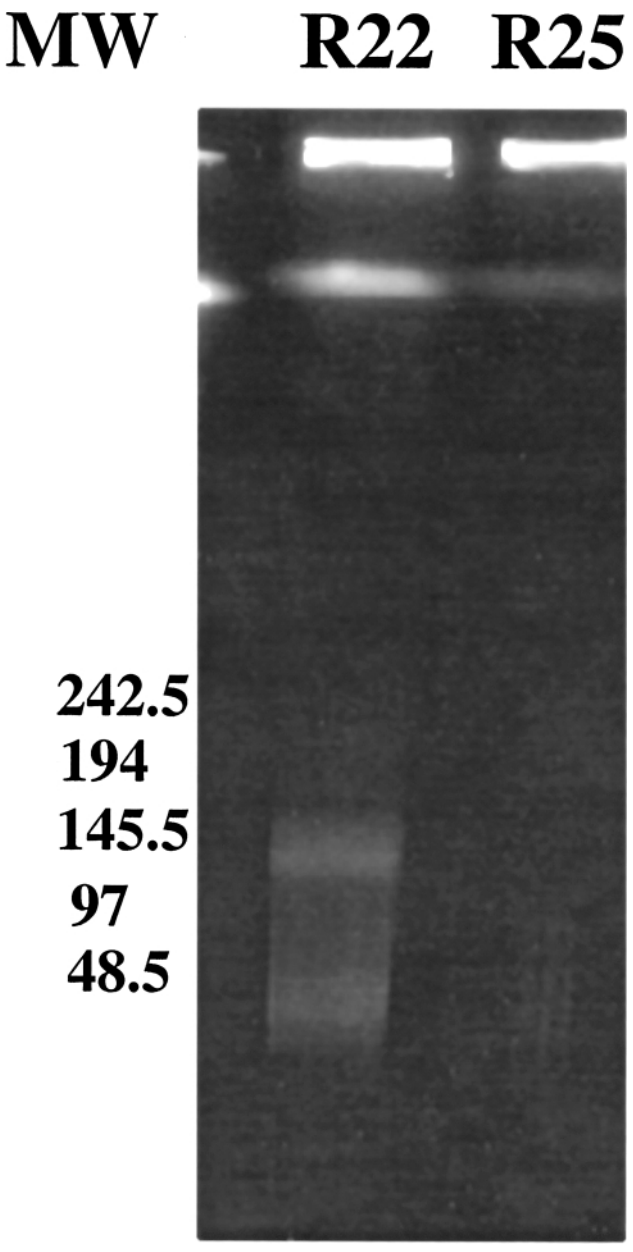

Figure 3 PFGE detection of large plasmids in strains R22 and R25. Lane marked MW is a λ concatamer molecular weight marker ladder and molecular weights are indicated on the left of this lane.

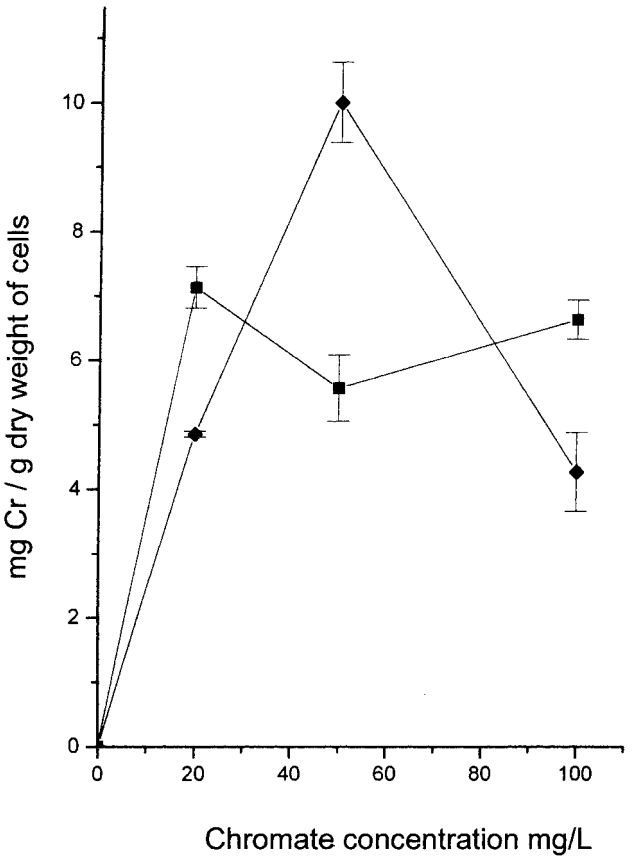

Figure 4 Whole cell accumulation of chromium after 72 h growth by strains R22 (◆) and R25 (■).

Discussion

Streptomyces sp. strains R22 and R25, two of the most promising strains selected in a previous screening program of metal resistance to cadmium, copper, and mercury [4], showed a high tolerance to chromate ions. The two strains showed similar profiles of growth inhibition at increasing concentrations of chromate. However, profiles of chromium accumulation were different in the two strains. Strain R22 displayed markedly higher chromium accumulation at a chromate concentration of 50 mg l^{-1} compared with concentrations of 20 and 100 mg l^{-1}. Strain R25 showed similar chromium accumulation at the three concentrations of chromate used.

16S rRNA sequence analysis revealed that, although strains R22 and R25 are both streptomycetes, they are not two strains of the same species nor are they closely related within the *Streptomyces* clade. An additional difference between strains R22 and R25 is that strain R22 harbors two large plasmids. Plasmids have often been implicated in metal resistance in a wide range of organisms (for reviews, see Refs. [8,31]). Plasmids have been clearly linked with mercury resistance in two *Streptomyces* spp. isolated from estuarine sediments [26]. The finding of large plasmids in strain R22 raises the interesting possibility that chromium resistance in this strain may be plasmid-mediated. The different profiles of chromium

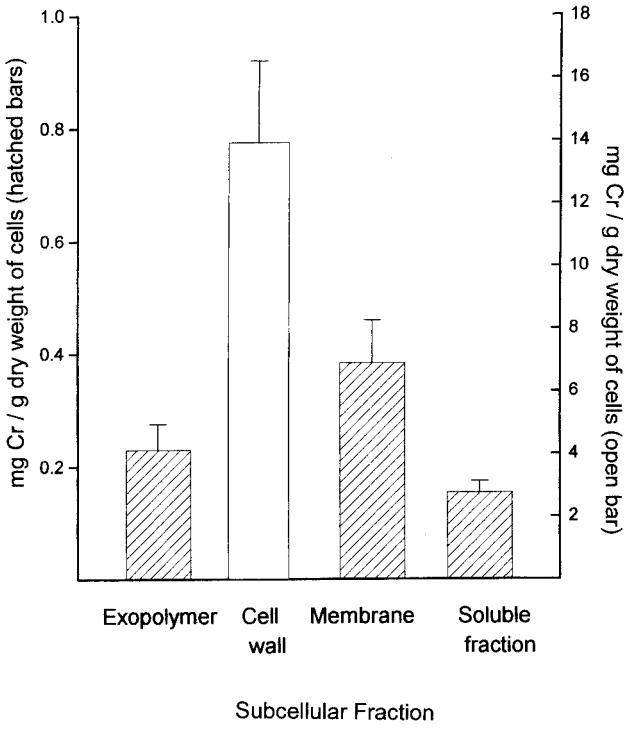

Figure 5 Chromium distribution in the subcellular components of cells of strain R22 grown at an initial chromium concentration of 50 mg l^{-1}. Cell wall chromium accumulation is shown by the open bar read on the right *y*-axis. Accumulation by other cellular components was at much lower levels and is indicated by hatched bars read on the left *y*-axis.

resistance shown by strains R22 and R25, the presence of plasmids in strain R22, and the fact that these two strains are fairly distantly related *Streptomyces* suggest that the mechanisms of chromium resistance and accumulation are not necessarily the same in these two strains.

In Gram-negative bacteria, both resistance to and reduction of chromate occur, but the resistance encoded by plasmids in these bacteria appears to be unrelated to chromate reduction, rather being due to reduced intracellular accumulation of chromate [18]. A recent study showed efflux of chromate from *Pseudomonas aeruginosa* cells by means of a chromate resistance protein [2]. Chromate reduction in Gram-negatives has been associated with both the cytoplasmic fraction [17,34] and cell membranes [36]. In Gram-positives, chromate reduction has been shown in *Bacillus* strain QC1-2. In this case, the Cr(VI) reductase enzyme was soluble rather than membrane-associated, and the resultant Cr(III) ions remained in the supernatant [7]. In actinomycetes, Aislabie and Loutit [1] showed that a coryneform isolate accumulated Cr(III) in extracellular polysaccharide material. In the only previous study in *Streptomyces* spp., chromate reduction enzyme activity in strain 3M was associated with a particulate cell fraction, probably containing cytoplasmic membranes, and chromate resistance and reduction were found to be unrelated [11]. In this study, the highest chromium accumulation in *Streptomyces* strain R22 was associated with the cell wall fraction. Insignificant chromium accumulation occurred in the membrane and ribosome fraction and the soluble fraction. Chromium accumulation by the cell wall may result from reduction of chromate anions (CrO_4^{2-}) to chromic ions (Cr^{+3}), a process previously reported by Llovera *et al.* [24]. The reduced Cr^{3+}, which exists as a cation, could be more amenable to complexation to the cell wall [28]. The low chromium concentration found in the exopolymer fraction of strain R22 could be the result of a negative interaction between chromate, which exists as an oxyanion at alkaline pH values, and anionic exopolymer material [15]. Reduction of Cr(VI) to Cr(III) and complexation of Cr(III) by the cell wall may together prevent the toxic effects of chromium and explain the growth of these strains at CrO_4^{2-} concentrations as high as 100 mg l^{-1}.

Isolation of the chromate-resistant, chromium-accumulating *Streptomyces* strains R22 and R25 provides additional actinomycete strains in which the mechanisms of chromium resistance and accumulation can be studied. In addition, these strains may have potential for bioremediation of chromium-contaminated soils, sediments, and waste streams. Genes encoding chromium resistance in these strains may be useful for introduction into related actinomycetes used to degrade other pollutants in order to produce strains capable of bioremediation of mixed wastes.

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